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(54) Title: METHODS AND COMPOSITIONS USING TUMOR SPECIFIC SOLUBLE INTERLEUKIN-2 RECEPTOR ALPHA MOLECULES

(57) Abstract

Provided are compositions comprising soluble IL-2Rα molecules (tsIL-2Rα) produced and shed by metastatic cells; nucleic acid molecules encoding tsIL-2Rα, vectors for expressing tsIL-2Rα; assay kits for detecting and quantitating the amount of tsIL-2Rα in a sample; and methods for detecting and quantitating the amount of tsIL-2Rα that may be present in a body fluid of an individual. A method for detecting the presence or absence of tsIL-2Rα in a body fluid of an individual comprises obtaining a sample of body fluid from the individual; contacting the sample with an affinity ligand which binds to an epitope on tsIL-2Rα; and detecting the amount of tsIL-2Rα bound to the affinity ligand.

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METHODS AND COMPOSITIONS USING TUMOR SPECIFIC SOLUBLE INTERLEUKIN-2 RECEPTOR ALPHA MOLECULES

FIELD OF THE INVENTION

The present invention relates to compositions comprising novel, tumor-specific molecules of soluble Interleukin-2 receptor alpha ("tsIL-2Rα"), and methods for detecting and measuring one or more species of tsIL-2Rα in the body fluid of an individual. The detection of one or more species of tsIL-2Rα in the body fluid of an individual can be used to aid in diagnosis of metastasis or metastatic recurrence; establish a metastasis prognosis; aid in the selection of therapeutic treatment, particularly directed to metastases; and monitor efficacy of therapeutic treatment of tumors, particularly metastatic cells.

BACKGROUND OF THE INVENTION

1. Metastasis.

Metastasis is the spread of malignant tumors to secondary sites remote from the original or primary tumor. Metastasis presents a cancer clinician with difficulty in diagnosing and treating the malignant tumor because (a) metastases may be comprised of as little as one or a few cells thereby evading clinical diagnosis even with modern techniques; (b) often metastases have already been seeded by the time a patient is diagnosed with a malignant non-lymphoid tumor (Silverberg et al., 1989, *CA Cancer J. Clin.* 39:3-21); (c) treatment is more complex than simple surgical excision of the primary tumor; (d) systemic therapy for metastatic non-lymphoid tumors, such as renal cell carcinoma (Rosenberg et al., 1985, *N. Engl. J. Med.* 313:1485-1492), remains ineffective with little survival advantage; and (e) not all malignant tumors have the same metastatic potential, and no soluble tumor-specific marker has been described for

determining whether any particular non-lymphoid tumor will develop metastasis.

2. IL-2 And The IL-2 Receptor.

2.1 IL-2 And IL-2R Interaction

IL-2 and the IL-2 receptor (IL-2R) interact in regulating the T cell immune response. The IL-2R is present in three forms classified by their binding affinity for IL-2 and by the different combinations of two binding proteins (the α and β chains). High affinity IL-2R contain both α and β chains, intermediate affinity contain β chains, and low affinity contain α chains (Leonard et al., 1990, *Prog. Clin. Biol. Res.*, 352:179-187). In T cell populations, IL-2Rα (also known as p55) is only expressed on activated T cells, and it has been shown that IL-2 exerts its T cell growth promoting effects via stimulation of the α chain.

2.2 Soluble IL-2Rα As A Marker For Inflammatory Diseases

Activated T cells hyperexpress IL-2R in a membrane bound form, and also shed soluble IL-2R in a form as soluble IL-2Rα (sIL-2Rα). The mature form of human IL-2Rα consists of 251 amino acids (amino acids 22-272 of SEQ ID NO:1) with a calculated molecular mass (M_r) of 28.428 kDa (kilodaltons) (Cosman et al., 1984, *Nature* 312 20/27:768-771). A M_r of about 53-55 kDa, when isolated from a cell, suggests that the receptor could be composed of 50% carbohydrate. The lymphocyte-derived sIL-2Rα is a truncated form of membrane bound sIL-2Rα, having a M_r in the range of 45-50 kDa, with the difference being primarily changes in the protein. It is believed that the release of sIL-2Rα involves proteolytic processing of the mature receptor resulting in the C-

terminal amino acid as Cys, at position 192 (amino acid 213 of SEQ ID NO:1; Robb et al., 1987, *J. Immunol.* 139:855-862).

Increased levels of sIL-2Rα have been detected in the serum of patients diagnosed with an inflammatory disease, including malignancy. Kung et al. (U.S. Patent No. 5,006,459) disclose the elevation of serum sIL-2Rα in patients with active lymphatic cancers such as leukemia and lymphoma; and viral infections. Further, the concentration of soluble IL-2Rα bears a direct relationship with the severity and prognosis of the lymphatic cancer. They also disclose that sIL-2Rα receptors were generally not elevated in patients with non-lymphatic cancers. It is disclosed that the levels of sIL-2Rα are elevated in clinical conditions characterized by increased T cell (malignant or normal) activation *in vivo*.

Other inflammatory diseases in which elevated levels of sIL-2Rα have been detected in the serum of affected patients include immune system disorders like allograft rejection (Colvin et al., 1987, *Clin. Immunol. Immunopathol.* 43:273-276); AIDS (Sethi et al., 1986, *Immunol. Lett.* 13:179-184); rheumatoid arthritis (Symons et al., 1988, *J. Immunol.* 141:2612-2618); subacute lupus erythematosus (Neish et al., 1993, *J. Dermatological Sci.* 5:143-149); pulmonary sarcodosis (Lawrence et al., 1988, *Am. Rev. Respir. Dis.* 137:759-764); and tuberculosis (Brown et al., 1989, *Am. Rev. Respir. Dis.* 139:1036-38). The elevated levels of sIL-2Rα in such diverse pathological conditions associated with lymphocyte cell activation, coupled with the fact that activated T cells shed sIL-2Rα, point to the elevated levels as being indicia of an immune response with lymphocytes as the source of such abnormally elevated levels of sIL-2Rα. In that regard, a positive correlation has been

shown between serum levels of IL-2Rα and lymphocyte activity in inflammatory diseases (Beckham et al., 1992, *J. Clin. Immunol.* 12:353-361; Hofmann et al., 1992, *Clin. Exp. Immunol.* 88:548-554; Carotti et al., 1994, *Rheumatol. Int.* 14:47-52).

Recently, elevated levels of sIL-2Rα have been detected in several types of solid tumors. Ginns et al. (1990, *Am. Rev. Respir. Dis.* 142:398-402) found elevated levels of sIL-2Rα in lung cancer patients. Those investigators had hypothesized that since activated T cells have been found in patients with lung cancer, and because sIL-2Rα apparently arises mainly from activated T cells, that elevated serum levels of sIL-2Rα might be found in lung cancer patients. Barton et al. (1993, *Blood* 81:424-429) report elevated serum and ascitic levels of sIL-2Rα in ovarian carcinoma patients. Those investigators conclude that since the most common cells of ascitic cellular infiltrate (besides tumor cells) are T cells and macrophages, and because activated T cells and macrophages express and shed sIL-2Rα, the major source of sIL-2Rα in the blood and ascites of ovarian carcinoma patients is activated T cells and macrophages. Murakami et al. (1994, *Cancer* 74:2745-2748) report elevated serum levels of sIL-2Rα in patients with gastric carcinoma, wherein higher levels were observed in patients with lymph node metastasis as compared to those without lymph node metastasis. Those investigators conclude that T lymphocytes, such as those stimulated by metastatic cancer cells, produce the high concentrations of sIL-2Rα observed. As summarized by Lissoni et al. (1990, *Eur. J. Cancer* 26:33-36), elevated levels of sIL-2Rα have also been observed in patients with other types of solid tumors, having either a primary tumor and/or metastatic

disease (depending on the tumor type). The mechanism for the enhanced production of sIL-2Rα was thought to be either an activation of the immune system or the expression of an immune dysfunction.

2.3 Expression Of Membrane Bound or Internal IL-2Rα By Solid, Non-lymphoid Tumors And Their Metastases

The present inventor, in U.S. Patent No. 5,536,642 (incorporated herein by reference), disclosed that primary tumors having a high potential to metastasize, and their metastases, express tumor cell-associated IL-2Rα. Using commercially available monoclonal antibodies (recognizing either exon 4 or exon 6) measurement of tumor cell-associated (either internal or membrane bound) IL-2Rα in experimental tumors and human tumors showed a correlation between tumor cell-associated expression of IL-2Rα and the metastatic potential of a primary tumor, i.e. the likelihood that the primary tumor has already, or will, metastasize. Also disclosed are methods for predicting the metastatic potential of solid, non-lymphoid tumors by measuring the tumor cell-associated IL-2Rα expression. While such diagnostic and prognostic methods are useful, they require a tumor specimen to measure the tumor cell-associated IL-2Rα. Unfortunately, for some tumor types, the primary tumor is inaccessible, or performing such a biopsy is clinically very dangerous. Further, depending on the tumor type, concern need be exercised regarding the possible presence of infiltrating activated T cells within the biopsy which can be a source of IL-2Rα. Lastly, since a biopsy is required, it must already be known that the individual is a tumor-bearing individual.

In sum, at the time of the invention it was thought that activated lymphocytes are the primary, if not

the only, source of sIL-2Rα in patients with solid, non-lymphoid tumors. Further, the background and related art does not disclose or correlate the existence of multiple species of tumor-specific soluble IL-2Rα (tsIL-2Rα) in patients with solid, non-lymphoid tumors or metatstatic cells. Nor does the background and related art disclose the use of tsIL-2Rα in diagnostic and prognostic methods for solid, non-lymphoid tumors or their metastases.

Hence, a need still exists for a relatively rapid, simple and efficient method for measuring tumor-specific IL-2Rα (distinguishable from IL-2Rα of lymphocyte origin), such as tsIL-2Rα. Such a method may aid in the early detection of cancer, whether it be the primary tumor and/or metastatic cells detected, in a manner which can distinguish between the malignancy and other inflammatory diseases. In general, the earlier cancer is detected, the better the chance of successful treatment and thus survival. In addition to having the potential to affect the survival of the patient, early detection of metastatic disease has other advantages. The cost of treating a tumor depends on its stage of progression, e.g., the cost of treating early stage breast cancer may range from $10,000 to $15,000; whereas, the cost of treating advanced metastatic disease may range from $150,000 to $175,000 (Carrera, 1995, *Denver Business J.* 46:3). And in the case where a primary tumor is surgically removed, a rapid, simple and efficient method for monitoring the levels of tumor-specific IL-2Rα may be a cost-effective alternative to the relatively high cost and inconvenience of post-therapeutic evaluations for residual or metastatic disease using radiographic techniques or imaging techniques (CT scans, NMR scans). Lastly, such a method would greatly facilitate the choice and mode of anticancer therapy, when, for example, metastases are suspected.

SUMMARY OF THE INVENTION

A primary object of the invention is to provide compositions comprising one or more tumor-specific IL-2Rα molecules.

Another primary object of the invention is to provide a method for detecting metastatic cells, or the existence of a primary tumor producing metastatic cells, by measuring levels of one or more species of tsIL-2Rα.

Another object of the invention is to provide a method for detecting a soluble marker in body fluids which is tumor-specific, and which can be used to distinguish between inflammatory diseases; and residual, recurrent or metastatic cancer.

A further object of the invention is to provide a method for staging malignant disease in an individual having solid, non-lymphoid tumors and/or metastatic cells by measuring levels of one or more species of tsIL-2Rα.

Another object of the invention is to provide a method for determining the metastatic potential of a non-lymphoid tumor by determining the level of expression of one or more species of tsIL-2Rα.

A further object of the present invention is to provide a method for monitoring the effectiveness of anticancer therapy against primary non-lymphoid tumors having a high probability of metastasis, or metastatic cells produced therefrom.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a plot of the amino acid sequence of SEQ ID NO:5 which graphically illustrates regions predicted to be antigenic.

FIG. 2 is a bar graph showing the increase in human sIL-2Rα as the tumor progresses *in vivo* over time.

FIG. 3 is a bar graph showing the increase in human sIL-2Rα in relation to number of cells injected.

FIG. 4 is a bar graph showing the increase in human sIL-2Rα in mice injected with the more metastatic SW620 (5×10^5 cells) than that in mice injected with SW420 (5×10^5 cells).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

"tsIL-2Rα" or "soluble IL-2Rα produced by metastatic cells" are terms used hereinafter for the purposes of the specification and claims to refer to a human tumor-specific soluble IL-2Rα (a) that may be encoded from cDNA, when copied transcribed from mRNA, lacking some or all of the nucleotides found within the region comprising nucleotide positions 750 to 862 of SEQ ID NO:8; and (b) has an amino acid sequence lacking some or all of the amino acid sequence found between residues 240 to 265 of SEQ ID NO:1. As will be more apparent from the following description, such a deletion of nucleotides can result in an encoded tsIL-2Rα comprising a protein of truncated form when compared to the amino acid sequence of IL-2Rα as depicted in SEQ ID NO:1. Alternatively, such a deletion of nucleotides can result in a tsIL-2Rα comprising a protein of a greater number of amino acids when compared to the amino acid sequence of IL-2Rα as depicted in SEQ ID NO:1. In either case, each species of tsIL-2Rα contains at its C-terminal portion at least one new epitope unique to the respective tsIL-2Rα (i.e., not immunologically cross-reactive with, nor shared by either the amino acid sequence of IL-2Rα as depicted in SEQ ID NO:1, and/or by soluble IL-2Rα produced T lymphocytes), as will be more apparent from the following descriptions. Addition-

ally, because the tsIL-2Rα lacks some or all of the transmembrane domain of IL-2Rα (the transmembrane domain represented between residues 240 to 265 of SEQ ID NO:1), tsIL-2Rα is shed or secreted from the metastatic cell which produces it. RNA or DNA for tsIL-2Rα, and indirectly the tsIL-2Rα molecules themselves, may be identified and distinguished and amplified by use of one or more oligonucleotides consisting of the nucleotide sequences depicted in SEQ ID NOs: 2, 3, or 4.

"Body fluids" is a term used hereinafter for the purposes of the specification and claims to refer to a body fluid of an individual into which tsIL-2Rα is shed by solid non-lymphoid tumors or metastatic cells. Particularly, "body fluids" include fluids normally assayed for a indicator of neoplastic disease, including but not limited to, blood or blood component (serum or plasma), and other fluids including ascitic fluid (e.g. in ovarian carcinoma patients; Barton et al., 1993, *Blood* 81:424-429), cerebrospinal fluid (CSF) (central nervous system or CNS tumors, e.g., cerebellar medulloblastoma; Salmaggi et al., 1994, *Int. J. Neurosci.* 77:117-125), urine (e.g., bladder tumors; Balbay et al., 1994, *Urology* 43:187-190); lymph fluid (e.g. tumor-involved lymph nodes; Vitolo et al., 1993, *Eur. J. Cancer* 29A:371-377), and pleural fluid (lung cancer, e.g. adenocarcinoma, squamous, small cell, and large cell carcinomas; Yamaguchi et al., 1990, *J. Lab. Clin. Med.* 116:457-461).

"Consisting of", in relation to amino acid sequence of a protein or peptide described herein, is a term used hereinafter for the purposes of the specification and claims to refer to a conservative substitution or modification of one or more amino acids in that sequence such that the tertiary configuration of the protein or peptide is

substantially unchanged. "Conservative substitutions" is defined by aforementioned function, and includes substitutions of amino acids having substantially the same charge, size, hydrophilicity, and/or aromaticity as the amino acid replaced. Such substitutions, known to those of ordinary skill in the art, include glycine-alanine-valine; isoleucine-leucine; tryptophan-tyrosine; aspartic acid-glutamic acid; arginine-lysine; asparagine-glutamine; and serine-threonine. "Modification", in relation to amino acid sequence of a protein or peptide, is defined functionally as a deletion of one or more amino acids which does not impart a change in the conformation, and hence the biological activity or specificity of induced antibody, of the protein or peptide sequence.

"Consisting of", in relation to a nucleic acid sequence described herein, is a term used hereinafter for the purposes of the specification and claims to refer to substitution of nucleotides as related to third base degeneracy. As appreciated by those skilled in the art, because of third base degeneracy, almost every amino acid can be represented by more than one triplet codon in a coding nucleotide sequence. Further, minor base pair changes may result in variation (conservative substitution) in the amino acid sequence encoded, are not expected to substantially alter the biological activity of the gene product. Thus, a nucleic acid sequencing encoding a protein or peptide as disclosed herein, may be modified slightly in sequence (e.g., substitution of a nucleotide in a triplet codon), and yet still encode its respective gene product of the same amino acid sequence.

The term "metastatic cell" is used herein, for purposes of the specification and claims, to mean cells which have metastasized, or in the process of metastasizing, from a solid, non-lymphoid tumor.

The term "affinity ligand" is used herein, for purposes of the specification and claims, to mean a molecule which has binding specificity and avidity for at least one antigenic epitope unique to tsIL-2Rα (i.e., not expressed by sIL-2Rα nor membrane bound IL-2Rα such as SEQ ID NO:1) wherein the molecule comprises one or more of a lectin; a monoclonal antibody (mAb); immunoreactive fragments produced or derivatives derived from mAb; peptides; and aptamers. Aptamers can be made against tsIL-2Rα epitopes using methods described in U.S. Patent No. 5,789,157 (the disclosure of which is herein incorporated by reference). Peptides can be made against tsIL-2Rα epitopes by using tsIL-2Rα to screen a phage display library using methods known to those skilled in the art (see, e.g., Smith and Scott, 1993, *Methods Enzymol.* 217:228) and/or a commercially available kit.

The term "monoclonal antibody" ("mAb"), is used herein, for purposes of the specification and claims, to mean a monoclonal antibody produced in an animal or by recombinant means or by means of genetic engineering. For example, a mAb may include one or more chimeric or genetically modified monoclonal antibodies which may be preferable for administration to humans. The term "monoclonal antibody" is also used herein, for purposes of the specification and claims, to include immunoreactive fragments or immunoreactive derivatives (e.g., peptides) derived from a mAb molecule, which retain all or a portion of the binding function of the whole mAb molecule. Such immunoreactive fragments or immunoreactive derivatives are known to those skilled in the art to include $F(ab')_2$, Fab', Fab, Fv, scFV, Fd', Fd, and the like. Methods for producing the various fragments from mAbs are well known in the art (see, e.g., Plückthum, 1992, *Immunol. Rev.* 130:152-188). For example, $F(ab')_2$ can be produced by pepsin digestion of the monoclonal

antibody, and Fab' may be produced by reducing the disulfide bridges of $F(ab')_2$ fragments. Fab fragments can be produced by papain digestion of the monoclonal antibody, whereas Fv can be prepared according to methods described in U.S. Patent No. 4,642,334. Single chain derivatives can be produced as described in U.S. Patent No. 4,946,778. The construction of chimeric antibodies is now a straightforward procedure (Adair, 1992, *Immunological Reviews* 130: 5-40,) in which the chimeric antibody is made by joining the murine variable region to a human constant region. Additionally, "humanized" antibodies may be made by joining the hypervariable regions of the murine monoclonal antibody to a constant region and portions of variable region (light chain and heavy chain) sequences of human immunoglobulins using one of several techniques known in the art (Adair, 1992, *supra;* Singer et al., 1993, *J. Immunol.* 150:2844-2857).

The term "nucleic acid amplification" is used herein, for purposes of the specification and claims, to mean one or more methods of amplifying nucleic acid sequences known to those skilled in the art. For example such methods include, but are not limited to: (a) polymerase chain reaction, which uses a thermostable DNA polymerase, and oligonucleotides primers, in a thermocycling process; (b) ligase chain reaction, utilizing DNA ligase and an oligonucleotide probe; (c) enzyme QB replicase, utilizing an RNA sequence template; and (d) nucleic acid sequence-based amplification.

The terms "solid, non-lymphoid tumor" or "non-lymphoid tumor" are used herein, for purposes of the specification and claims, to mean any tumor of ductal epithelial cell origin, including, but not limited to, tumors originating in the liver, lung, brain, lymph node, adrenal gland, breast, colon, pancreas, stomach, prostate, or reproductive tract (cervix, ovaries, endometrium etc).

The term "individual" is used herein, for purposes of the specification and claims, to mean a mammal. In a preferred embodiment, the mammal is a human.

The present invention relates to the unexpected finding that in addition to expressing cell associated IL-2Rα, malignant cells produce one or more soluble, tumor-specific forms of IL-2Rα. Thus, the present invention is directed to the detection and identification of tumoral-specific variants of IL-2Rα which are expressed in a soluble form by metastatic cells in blood or other body fluids. Such variants comprise species of tsIL-2Rα that can be identified and distinguished using one or more of oligonucleotide primers comprising SEQ ID NOs:2-4. The tsIL-2Rα species share essentially the same or similar first 235 or more amino acids in their respective amino acid sequences with the first 235 or more amino acids in IL-2Rα of lymphocyte or mononuclear cell origin depicted in SEQ ID NO:1. After that point in the amino acid sequences, the tsIL-2Rα species vary significantly from that of the carboxy terminus (e.g., the sequence of amino acids from about amino acid 235 to about amino acid 272) of IL-2Rα depicted in SEQ ID NO:1. With such variations, the tsIL-2Rα species have amino acid sequences in which amino acids occurring after about residue 235, and more particularly after about residue 240, may differ (a) antigenically, (b) functionally, and (c) compositionally when compared to sIL-2Rα of lymphocyte or mononuclear cell origin (amino acids 22-213 of SEQ ID NO:1) or cell associated IL-2Rα of lymphocyte origin or mono-nuclear cell origin, or cell associated IL-2Rα that may be produced by tumors (amino acids 22-272 of SEQ ID NO:1). Further, the present invention is directed to the detection

and quantitation of one or more species of tsIL-2Rα, and the use of such measurements in diagnostic and prognostic methods for identifying or treating metastatic cells. The measurement of such molecules can be used to aid in the diagnosis of metastases; to determine the metastatic potential of solid, non-lymphoid tumors; to stage the malignant disease; and to monitor the efficacy of anticancer therapy against metastatic cells expressing tsIL-2Rα.

EXAMPLE 1

This embodiment relates to the identification of human tsIL-2Rα. It was unexpectedly found in the discovery and development of the invention, that metastatic cells (including SW620) express and shed soluble IL-2Rα, in addition to express-ing a membrane bound (tumor cell-associated) form as disclosed in U.S. Patent No. 5,536,642, when grown in culture *in vitro* or when grown in an animal model system (athymic nude mice maintained in pathogen-free conditions). In general, and as described herein, the degree of metastatic behavior of the clonal cancer cell type correlated with the degree of shedding of soluble IL-2Rα; i.e., a highly metastatic clonal cell type sheds a higher amount of sIL-2Rα compared to a clonal cell type considered to exhibit a lesser degree of metastasis. To determine if the sIL-2Rα expressed by human metastatic cells was tumoral-specific or was identical to the sIL-2Rα of lymphocyte origin, the following studies were performed. A human lymph node metastasis of a colon adenocarcinoma cell (SW620; ATCC Accession No. 227-CCL) was used as a representative source of species of tsIL-2Rα, although any human metastatic carcinoma may be used as a source of human tsIL-2Rα (See

Example 3). SW620 cells are considered to be relatively highly metastatic.

SW620 cells were maintained in culture medium (Dulbecco's modified Eagle medium or Leibovitz's L-15 medium) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. Cells were harvested by centrifugation, washed, and total RNA was extracted. Briefly, media was removed from confluent 75 cm^2 flasks containing cultured SW620 cells. Ten milliliters of sterile phosphate buffered saline (PBS) was added to wash the cells, and then the PBS was removed. Three milliliters of trypsin-EDTA was then added to the flask and allowed to incubate at 37 C for 10 minutes. The cells were then removed from the flask by adding 10 ml of PBS, and pipeting the solution up and down until all the cells had detached from the flask. Cells were then added to a 50 ml conical tube containing 5 ml of complete culture media. The cells were then counted. Once the cell count had been determined, the cells were then centrifuged at 1200 rpm for 8 minutes at 4°C. The supernatant was then removed, and the cell pellet was then resuspended in a lysing reagent (Trizol reagent; 1 ml per $5x10^6$ to $1x10^7$ cells). The solution was mixed several times, and then 1 ml aliquots were added to sterile, RNASE-free microcentrifuge tubes. A 1 ml syringe with a 26 gauge needle is then used to aspirate each of the 1 ml samples twice in order to shear the DNA contained in the lysed cell suspension. The tubes are then allowed to incubate for 5 minutes at room temperature to permit dissociation of nucleotide complexes. Chloroform was then added, 200 µl, to each tube. The tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. The samples were then centrifuged for 15 minutes at 6500 rpm at 4°C. The RNA, found in the top aqueous layer, was transferred to a fresh tube. The

RNA was precipitated by adding 500 µl of isopropanol per tube. The tubes were allowed to incubate at room temperature for 10 minutes. Tubes were centrifuged for 15 minutes at 4°C at 6500 rpm. The supernatant was then removed, and the pelleted RNA was left at the bottom of the centrifuge tube(s). A total volume of 100 µl depc treated water was used to resuspend the pelleted RNA. The total RNA extracted was then quantitated by using the spectrophotometer. DNase I digestion of RNA preparations was carried out as shown in Table 1:

Table 1

Components	Amounts
total RNA	4-6 µg/reaction tube
10X reaction buffer	1 µl
Amplification grade DNase I	1 µl
DEPC-treated water	up to total volume of 10 µl

The 10X reaction buffer comprised 200 mM Tris-HCl (pH 8.4),500 mM KCl, and 20mM $MgCl_2$. The tube(s) containing the above components were incubated for 15 minutes at room temperature. To each tube, 1 µl of 25mM EDTA was added. Incubation was then carried out at 65°C for 15 minutes to heat inactivate the DNase I, then placed on ice for 1 minute. Collection of the reaction was done by brief centrifugation. This mixture was then directly used for reverse transcription.

cDNA was synthesized from total RNA using reverse transcriptase. RNA/primer tubes were setup as illustrated in Table 2.

Table 2

Components	Sample	No RT Control	Control
RNA			
1-5 µg total RNA	n µl	n µl	---
control RNA (50ng/µl)	---	---	1 µl
oligo(dT)12-18 primer	1 µl	1 µl	1 µl
DEPC-treated water	to 12 µl	to 12 µl	to 12 µl

These tubes were incubated for 10 minutes at 70° C, and then incubated on ice for at least 1 minute. The following tubes are setup for each reaction tube. The RT master mix components are added in the order stated: 10X PCR buffer, 2 µl; 25 mM $MgCl_2$, 2 µl; 10 mM dNTP (dATP, dCTP, dGTP, dTTP) mix, 1 µl; and 0.1M DTT, 2 µl. Seven microliters of the RT master mix was then aliquoted to each of the RNA/primer tubes described above. The tubes were then incubated at 42°C for 5 minutes. The reverse transcriptase was then added to each tube in 1 µl amounts, and incubated for 50 minutes at 42°C. The reaction was terminated by incubation of tubes at 70°C for 15 minutes, followed by chilling on ice. The reactions were collected by brief centrifugation, and then 1 µl of RNAse H was added to each tube. The tubes were incubated for 20 minutes at 37°C in order to digest away all RNA templates and leave only the cDNA.

cDNA was then amplified by nucleic acid amplification. An instrument for performing PCR (polymerase chain reaction) was used to amplify the cDNA corresponding to a portion of each respective species of tsIL-2Rα, as well as positive and negative control cDNA. A working volume of 50 µl was used in the reactions for detecting the presence of the specific target cDNA, and a 100 µl volume was used to amplify larger quantities for sequencing. For detection of

the desired target cDNA sequence (e.g., respective species of tsIL-2Rα, positive control cDNA, and negative control cDNA) specific primers for the target cDNA sequence were added in a reaction tube with 5 µl 10X PCR buffer, 3 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTP mix, 1 µl of the 3′ primer, 1 µl of the 5′ primer, 0.5 µl DNA polymerase (Taq; 2-5units/µl), 2 µl cDNA, and 36.5 µl ddH20. The reaction mixture was added to thin walled PCR tubes and run on the following PCR program: 95°C for 2 minutes; 2.5 cycles per second to 94°C; 94°C for 1 minute; 2.5 cycles per second to 45°C; : 45°C for 2 minutes; 2.5 cycles per second to 74°C; : 74°C for 1 minute; 2.5 cycles per second (39 times); 72°C for 7 minutes; 2.5 cycles per second to 4°C; and storage at 4°C.

EXAMPLE 2

This embodiment relates to the characterization of human tsIL-2Rα variants. Using the cDNA amplification procedures according to Example 1, amplified cDNA from several species of tsIL-2Rα were detected by agarose gel electro-pohoresis. Using SEQ ID NO:2 as 5′ primer, and SEQ ID NO:3 as a 3′ primer, a number of bands corresponding to portions of the nucelotide sequences of species of tsIL-2Rα were identified. In that regard, and as shown by agarose gel electrophoresis with ethidium bromide staining using a 2% high resolution agarose gel, using SEQ ID NO:2 and SEQ ID NO:3 as primers resulted in an amplified product of about 300 base pairs (bp), and an amplified product of about a 280 bp; as well as an amplified product of about 600 bp. Using SEQ ID NO:2 as 5′ primer, and SEQ ID NO:4 as a 3′ primer, another band corresponding to a portion of the nucelotide sequence of a species of tsIL-2Rα was identified. In that

regard, using SEQ ID NO:2 and SEQ ID NO:4 as primers, a 300 bp product and a 270 bp product were identified.

Based on this information, one species of tsIL-2Rα is a protein of approximately 338 amino acids (the mature form expected to be approximately 318 amino acids) consisting of the amino acid sequence depicted as SEQ ID NO:5. Sequence analysis software was used to determine the correct open reading frame, codon usage, base composition analysis, deduced amino acid composition, and predicted antigenicity of this species tsIL-2Rα. The deletion of nucleotides seen at the level of the cDNA of this particular species of tsIL-2Rα has particular significance when translated to the protein level. While SEQ ID NO:1 shows an amino acid sequence encoding an IL-2Rα molecule having a calculated molecular mass (M_r) of 28,428 daltons, the deduced amino acid sequences of the tsIL-2Rα depicted in SEQ ID NO:5 has a calculated M_r in excess of 31,000 daltons. Additionally, the amino acid sequence shows an additional N-glycosylation motif (around amino acid 267) as compared to the IL-2Rα amino acid sequence of SEQ ID NO:1. Importantly, sequence analysis confirms that the protein illustrated in SEQ ID NO:5 lacks a transmembrane binding domain; but importantly contains regions predicted to be highly antigenic (amino acids 245 to 255; 256 to 280; 280 to 290; 315 to 325; 330 to 338; see FIG. 1). These findings suggest that the mechanism behind the generation of soluble tumor-specific IL-2Rα may be alternative RNA splicing occurring in tumors, rather than arising from proteolytic fragmentation of membrane bound receptors. Of clinical significance is that the deletion of nucleotides from the tsIL-2Rα transcripts results in a frameshift mutation in the amino acid sequence of the respective tsIL-2Rα variants.

Another species of tsIL-2Rα may be a protein of approximately 247 amino acids (the mature form expected to be approximately 227 amino acids) consisting of the amino acid sequence depicted as SEQ ID NO:6. Sequence analysis software was used to determine the correct open reading frame, codon usage, base composition analysis, deduced amino acid composition, and predicted antigenicity of this species tsIL-2Rα. The deletion of nucleotides seen at the level of the cDNA of this particular species of tsIL-2Rα has particular significance when translated to the protein level. While SEQ ID NO:1 shows an amino acid sequence encoding an IL-2Rα molecule having a calculated molecular mass (M_r) of 28,428 daltons, the deduced amino acid sequences of the tsIL-2Rα depicted in SEQ ID NO:6 has a calculated M_r of about 22,700 daltons. Importantly, sequence analysis confirms that the protein illustrated in SEQ ID NO:6 lacks a transmembrane binding domain; but importantly contains a region comprised of a tsIL-2Rα-specific epitope predicted to be antigenic (comprising the joinder of exon 6 to exon 8; e.g. an epitope located between about amino acid 236 to about amino acid 243 of SEQ ID NO:6). These findings also suggest that the mechanism behind the generation of soluble tumor-specific IL-2Rα may be alternative RNA splicing occurring in tumors, rather than arising from proteolytic fragmentation of membrane bound receptors.

Another species of tsIL-2Rα may be a protein of approximately 249 amino acids (the mature form expected to be approximately 229 amino acids) consisting of the amino acid sequence depicted as SEQ ID NO:7. Sequence analysis software was used to determine the correct open reading frame, codon usage, base composition analysis, deduced amino acid composition, and predicted antigenicity of this species tsIL-2Rα. The deletion of nucleotides seen at the level of

the cDNA of this particular species of tsIL-2Rα has particular significance when translated to the protein level. While SEQ ID NO:1 shows an amino acid sequence encoding an IL-2Rα molecule having a calculated molecular mass (M_r) of 28,428 daltons, the deduced amino acid sequences of the tsIL-2Rα depicted in SEQ ID NO:7 has a calculated M_r of about 22,900 daltons. Importantly, sequence analysis confirms that the protein illustrated in SEQ ID NO:7 lacks a transmembrane binding domain; but importantly contains a region comprised of a tsIL-2Rα-specific epitope predicted to be antigenic (comprising the joinder of exon 6 to exon 8 with amino acids Val and Ala therebetween; e.g. an epitope located between about amino acid 236 to about amino acid 246 of SEQ ID NO:7).

EXAMPLE 3

This embodiment relates to a method for identifying human tsIL-2Rα variants. Using the illustrative methods and teachings according to the present invention, specifically according to Examples 1 and 2, one or more other human tsIL-2Rα variants may be identified and sequenced. From the teachings of the present invention, it would be apparent to those skilled in the art that human tsIL-2Rα, of sequences other than disclosed herein, may exist which differ at the mRNA level (or cDNA level) only in the number of nucleotides deleted from the region between nucleotide positions 750 to 862 of SEQ ID NO:8 (human IL-2Rα represented in cDNA form); and correspondingly, in the amino acid sequence by the proportion of the deletion in the third structural region (comprising the transmembrane domain) of the respective tsIL-2Rα molecule, and any additional amino acids caused by a frameshift and resultant usage of a

different stop codon. Any other human tsIL-2Rα that may exist which differ in sequence from sIL-2Rα (e.g. of lymphocyte origin) are functional equivalents of human ts-IL2Rα in the sense that they may be used in the diagnostic and prognostic methods disclosed in the present invention. That is, the difference in mRNA sequence (and/or cDNA sequence) can be used to generate primers for an assay based on nucleic acid amplification and detection of the amplified product; whereas, a difference in the amino acid sequence of the molecule can be used to generate ts-IL2Rα-specific antisera which can be used in an *in vitro* immunoassay.

As discussed previously herein, increased levels of sIL-2Rα have been detected in patients having inflammatory diseases including malignancy comprising solid non-lymphoid tumors. Further, the present invention shows that, unexpectedly, human tumors contribute to the pool of soluble IL-2Rα molecules by shedding ts-IL2Rα. To illustrate a method for identifying functionally equivalent human tsIL-2Rα variants, a source of tsIL-2Rα is required. As disclosed in U.S. Patent No. 5,536,642, tumor cell-associated IL-2Rα form has been found expressed by metastatic cells from many types of human non-lymphoid tumors. Selection of a human solid non-lymphoid tumor or non-lymphoid tumor cell line as a source of tsIL-2Rα is not particularly dependent upon the tumor type per se, as many different human solid non-lymphoid tumors can be used to isolate tsIL-2Rα. Rather, selection is dependent on demonstrating that such human tumor expresses IL-2Rα, wherein a proportion of that IL-2Rα comprises tsIL-2Rα. However, in general the higher the amount of IL-2Rα expressed by such a human tumor, the more desirable such human tumor is as a source of tsIL-2Rα.

In one illustration of this embodiment, total RNA is isolated and purified from a human solid non-lymphoid tumor either suspected of expressing IL-2Rα or demonstrated as expressing a high amount of IL-2Rα. Alternatively, mRNA is isolated and purified. Amplification of the tsIL-2Rα transcripts can be achieved by reverse transcription into cDNA, followed by nucleic acid amplification using IL-2Rα specific primers as described previously herein or using similar techniques known to those skilled in the art. Generation of amplified nucleic acid sequences which represent the tsIL-2Rα-specific sequences, allows for sequencing of the amplified nucleic acid sequences and for comparing the nucleotide sequence and/or the deduced amino acid sequence with the respective sequences of the tsIL-2Rα variants and sIL-2Rα disclosed herein. In another illustration of this embodiment, and using methods known to those skilled in the art, the cDNA that is generated may be inserted into a vector, such as a plasmid vector, to facilitate sequencing. Once the tsIL-2Rα variant nucleotide sequence is determined, amino acid sequence deduced, analysis of the tsIL-2Rα variant sequence can then performed with special software to determine if any frame-shift mutations detected result in antigenic epitopes unique to tsIL-2Rα (i.e., not shared by the mature form of IL-2Rα or sIL-2Rα of lymphocyte origin). Alternatively, peptides can be generated, as described below, which can be used to induce anti-IL-2Rα which antisera can be tested against sIL-2Rα of lymphocyte origin and the tumor shed sIL-2Rα to determine if the tumor shed sIL-2Rα is tsIL-2Rα.

EXAMPLE 4

This embodiment relates to a method for inducing anti-tsIL-2Rα antisera (polyclonal or monoclonal) for use in applications including, but not limited to, diagnostic and prognostic methods for detecting metastatic cells that may be present in a sample of body fluid from an individual. In one illustration of this embodiment, the entire tsIL-2Rα molecule can be used as an immunogen to induce anti-tsIL-2Rα antisera. However, since the tsIL-2Rα variant will likely also share one or more epitopes with sIL-2Rα (of lymphocyte origin) or IL-2Rα within its first approximately 240 amino acids, a preferred embodiment is to synthesize a peptide which comprises the amino acid sequences comprising one or more antigenic epitopes unique to the tsIL-2Rα variant ("tsIL-2Rα-specific epitopes"; e.g., not shared by or crossreactive with sIL-2Rα or IL-2Rα). Such peptides are synthesized as one, or may be chemically-linked if antisera is desired for more than one antigenic epitope unique to tsIL-2Rα variant(s). Antigenic sites of a protein may vary in size but can consist of from about 7 to about 15 amino acids. Thus, in synthesizing a peptide to comprise an antigenic epitope unique to the tsIL-2Rα variant, the size of the peptide synthesized should comprise at least from about 7 to 15 amino acids, and may comprise more than 15 amino acids. Such peptides can be synthesized using one of the several methods of peptide synthesis known in the art including standard solid peptide synthesis using tert-butyloxycarbonyl amino acids (Mitchell et al., 1978, *J. Org. Chem.* 43:2845-2852), using 9-fluorenylmethyl-oxycarbonyl amino acids on a polyamide support (Dryland et al., 1986, *J. Chem. So. Perkin Trans. I*, 125-137); by pepscan synthesis (Geysen et al., 1987, *J. Immunol. Methods* 03:259; 1984,

Proc. Natl. Acad. Sci. USA 81:3998); by standard liquid phase peptide synthesis; or by recombinant expression vector systems (insertion of the nucleic acid sequence encoding the peptide into an expression vector, transforming the appropriate host cell system for expression with the recombinant expression vector, and then affinity purifying the peptide produced recombinantly using methods known in the art). Modification of the peptides, such as by conservative substitution of amino acids (and including extensions and additions to amino acids) and in other ways, may be made so as to not substantially detract from the immunological properties of the peptide. Illustrative examples of peptides which may be used for inducing anti-tsIL-2Rα antisera may include: for SEQ ID NO:5, as shown in FIG. 1, a peptide comprising amino acids 242 to 338; for SEQ ID NO:6, a peptide comprising an epitope formed by the joinder of exon 6 to exon 8 (e.g., a peptide comprising an epitope located between from about amino acid 236 to about amino acid 243); and for SEQ ID NO:7, a a peptide comprising an epitope located between from about amino acid 236 to about amino acid 246).

In another related embodiment, the peptide may be synthesized to include additional amino acid sequences comprising a T-cell epitope. T-cells play a major role as helper cells for efficient antibody production. A T-cell epitope promotes specific helper T-cell responses in generating a efficient and effective immune response, and can overcome genetic restrictions in an immune response to an antigen, thereby broadening the effective response in a large number of genetically diverse individuals. Inclusion, as part of the peptide synthesized, of a T-cell epitope may result in an enhanced immune response compared to a peptide lacking the T-cell epitope. T-cell epitopes having defined sequences which can be incorporated as part of a synthesized

peptide, as understood by those skilled in the art, include, "promiscuous" T-cell epitopes from tetanus toxin (Panina-Bordignon et al., 1989, *Eur. J. Immunol.* 19:2237-2242; Ho et al., 1990, *Eur. J. Immunol.* 20:477; Kaumaya et al., 1993, *J. Mol. Recognition*, 6:81-94; all herein incorporated by reference); and from measles virus (Partidos and Stewart, 1990, *J. Gen. Virol.* 71, 2099-2105; herein incorporated by reference); and "universal" T-cell epitope peptides (T2, T4, and T6) from bacterial outer membrane protein TraT (Russell-Jones, 1993, *Vaccine* 11:1310, herein incorporated by reference).

The peptide comprising the at least one antigenic epitope specific to an tsIL-2Rα variant(s), or the at least one antigenic epitope specific to tsIL-2Rα variant(s) plus a T-cell epitope, can be used as an immunogen to generate anti-tsIL-2Rα antisera (polyclonal or monoclonal). Polyclonal antisera is generated using methods known to those skilled in the art, and involves repeated immunizations of an individual with a preparation of an immunologically-effective amount of the peptide with or without an adjuvant; harvesting the serum of the immunized individual; and immunopurifying from the serum the anti-tsIL-2Rα antisera induced. Monoclonal antibodies to the peptide can be induced using methods known to those skilled in the art. Murine monoclonal antibodies may be generated by injecting mice two or three times with an immunologically-effective amount of the peptide (or with tsIL-2Rα) with or without an adjuvant; harvesting the primed spleen cells from the immunized mice; fusing the spleen cells with a mouse myeloma cell line; selecting for the fused cells; isolating clones of the fused cells; and screening the clones by an immunoassay for immunoreactivity with the peptide (or with tsIL-2Rα).

Alternatively, for various applications, it may be desirable to generate human monoclonal antibodies which comprise anti-tsIL-2Rα antisera. Such applications include use of the human monoclonal antibodies in radioimmunodetection and radioimmunotherapy in humans (Chaudhuri et al., 1994, *Cancer* 73: (3 Suppl):1098-1104; herein incorporated by reference). Human monoclonal antibodies can be generated *in vitro* by fusion of mutant myeloma cells with human lymphoid cells immunized with purified peptide or peptide plus adjuvant (Chaudhuri et al., 1994, *supra*); by affinity selection from an human antibody library expressed on the surface of filamentous phage (Srikantan et al., 1994, *AIDS* 8:1525-32); or immunization of human peripheral blood lymphocytes (PBL) with subsequent Epstein-Barr virus infection (Chin et al., 1994, *Immunology* 81:428-434). Human monoclonal antibodies can be generated *in vivo* by using an immunogen comprising the purified peptide or tsIL-2Rα (with or without adjuvant) for vaccination of human PBL-SCID mice (Walker et al., 1994, *Immunology* 83:163-170; Chargui et al., 1995, *J. Immunol. Methods* 181:91-100).

EXAMPLE 5

This embodiment illustrates that human tumor cells secrete tsIL-2Rα.. A human metastatic tumor cell line was analyzed by confocal microscopy for secretion of tsIL-2Rα by staining with labeled anti-IL-2Rα antibody. tsIL-2Rα secretion was visualized as evident by the stained granules seen blebbing from the membrane. For evidence of *in vivo* tsIL-2Rα secretion, a SCID mouse model system was used to implant human carcinoma cell lines in the spleens of mice to allow for growth and metastases of these cells. Two groups were injected with cell line SW620 (very metastatic); one group with 5×10^5 cells, one group with 2×10^6. Another

group was injected with SW420 (less metastatic)- 5 x 10^5 cells. At 7 days, 14 days, and 21 days after injection, serum samples were obtained and assayed by ELISA for tsIL-2Rα secreted by the human tumor cells. The amount of tsIL-2Rα secreted by human tumor cells appears to be correlated with tumor progression. FIG. 2 is a bar graph showing the increase in human tsIL-2Rα as the tumor progresses *in vivo* over time. After 21 days, the amount of tsIL-2Rα detected was approximately 6-fold (90 pM) higher than that detected at 7 days (15 pM). Additionally, the amount of human tsIL-2Rα appears to be correlated with tumor cell volume. FIG. 3 is a bar graph showing the increase in human tsIL-2Rα in relation to number of cells injected. After 21 days, tsIL-2Rα detected was 2-fold (180 pM) higher with an injection of 4-fold more cells (2 x 10^6) than that detected at 21 days (90 pM) with an injection of 5 x 10^5 cells.

The amount of human tsIL-2Rα also appears to be correlated with metastasis. FIG. 4 is a bar graph showing the increase in human tsIL-2Rα in mice injected with the more metastatic SW620 (5 x 10^5 cells) than that in mice injected with SW420 (5 x 10^5 cells). Total splenectomy of SCID mice bearing tumors (72 hours after injection) resulted in complete clearance of tsIL-2Rα from the serum within 7 days; thereby confirming the relationship between the presence of tumor cells and tsIL-2Rα expression. The presence of dormant tumor cells in various organs within the tumor bearing SCID mice was assayed using an *Alu*-based nucleic acid amplification method of detection. Human *Alu* sequences were used as primers in polymerase chain reaction (and confirmed using primers specific for the human Y chromosome). No amplified products were apparent in the organs of control uninjected SCID mice nor the SCID mice injected with SW420. However,

amplified products were seen in the spleen, bone marrow, kidney, lungs, brain, and heart of SCID mice injected with SW620, despite no anatomical signs of metastasis being present. Thus, for the more metastatic human colon carcinoma cells, presence and dissemination in different organs of SCID mice correlates with the course of detection of tsIL-2Rα. Lymphocytes have been reported to be the main source of sIL-2Rα in cancer patients (activation of T-lymphocytes). These results indicate that metastatic cells themselves are an alternative, if not a primary source, of serum soluble IL-2Rα in cancer patients.

Using primers SEQ ID Nos: 2-4, either mRNA or commercially available cDNA from normal (non-cancerous) human tissues was processed and examined for nucleic acid sequences corresponding to those of tsIL-2Rα. Such tsIL-2Rα sequences were not detectably present in human adrenal gland tissue, human bone marrow tissue, and human lung tissue; thereby supporting the sequences as having specificity for nucleic acid molecules related to or encoding tsIL-2Rα.

EXAMPLE 6

This embodiment relates to methods of using anti-tsIL-2Rα antisera in immunoassays for diagnostics; and for prognostic and staging, in detecting tsIL-2Rα shed in body fluids by metastatic cells.

6.1 Immunoassays for tsIL-2Rα

In a preferred method of this embodiment, the presence or absence of tsIL-2Rα in a body fluid can be quantitated by using a specific antibody (anti-tsIL-2Rα) to assay for the physical presence of tsIL-2Rα. In one mode of this embodiment, tsIL-2Rα present in the body fluid may be

used as an antigen in immunoassays designed to detect or quantificate tsIL-2Rα. For most body fluids, such as blood, ascitic fluid, CSF, urine, lymph fluid, and pleural fluid, it may be desirable to centrifuge the body fluid after collection to remove cells and debris that could potentially interfere with the sensitivity of the assay. A sample of the body fluid may then be used as a source of antigen in any immunoassay system known in the art including, but not limited to, radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), "sandwich" assays, precipitin reactions, agglutination assays, and fluorescent immunoassays.

Because tsIL-2Rα appears primarily, if not exclusively, of metastatic cell origin, and thus not normally found in body fluid which do not contain nor is in contact with metastatic cells, detection of a level of tsIL-2Rα (above the level of the negative control used to establish a level of background detection in the assay) may be indicative of the presence of metastatic cells in the individual whose sample of body fluid was tested. It has been observed during the development of the present invention that factors which affect the measurable levels of human tsIL-2Rα include a non-lymphoid tumor's metastatic potential (e.g., a beginning process of metastasis); existence of metastases; the number of metastases, tumor volume; progression of metastatic disease, e.g., advances in the development of metastases; and the nature of metastatic cells' milieu, including contact with surrounding body fluids. As described in Example 5 herein, tumor expression of tsIL-2Rα is associated with metastatic behavior; i.e. metastatic cells. Thus, for example, serum tsIL-2Rα levels correlate with the number of metastatic cells, or primary tumor cells producing metastatic cells. The greater number of such cells, the more elevated the levels of tsIL-2Rα

detected. Further, the degree of metastatic potential or malignancy has been shown to correlate with the amount of tsIL-2Rα expressed or shed. For example, SW620 is more metastatic than SW480 (see Table 1), and has been observed to shed an increased amount of tsIL-2Rα compared to SW480. Additionally, tsIL-2Rα can be detected in body fluid, even if primary tumor is absent, with the existence of micro-metastases which secrete tsIL-2Rα.

In one preferred illustration of this embodiment, tsIL-2Rα levels are measured by an enzyme-linked immuno-sorbent assay (ELISA). The human body fluid collected may be centrifuged to remove cells and debris. In one illustration, a sample of the body fluid is contacted with a first affinity ligand used to bind to and capture tsIL-2Rα that may be present in the sample, and then a second affinity ligand is added to the assay system to detect any tsIL-2Rα that may be bound to the first affinity ligand. The second affinity ligand may be conjugated to a detectable moiety so as to allow detection of any bound tsIL-2Rα in the assay system, or by the subsequent addition of a substrate for detection of any bound tsIL-2Rα in the assay system. Alternatively, a secondary antibody which is conjugated to a detectable moiety is used to bind to the second affinity ligand, if present in the assay system. In one embodiment, the first affinity ligand binds specifically to tsIL-2Rα (e.g., not to sIL-2Rα nor IL-2Rα), wherein the affinity ligand is immobilized to a reaction surface (such as bound to the well of a microtiter plate). After an incubation, the reaction surface is washed, added into the assay is a second affinity ligand recognizing human IL-2Rα (e.g., anti-human IL-2Rα monoclonal antibody to sIL-2Rα) which recognizes an epitope common to both tsIL-2Rα and sIL-2Rα

(and/or IL-2Rα). This second affinity ligand may be conjugated to a detectable moiety (or alternatively, a secondary antibody used to bind to the second affinity ligand, may be labeled with the detecable moiety). Such detectable moieties are known in the art to include, but are not limited to, a fluorochrome, chromophore, or enzyme. Examples of such detectable moieties include alkaline phosphatase, fluorescein-5-isothiocyanate, peroxidase, phycoerythrin, and rhodamine, magnetic beads, or refractive beads. Depending on the detectable moiety used, a substrate may be required to interact with the detectable moiety to generate a detectable and measurable signal. After a the addition of the second affinity ligand, the reaction surface is washed, and then assayed for the detectable amount of detectable moiety which correlates with the presence (and amount) or absence of tsIL-2Rα in the sample tested. An additional wash step and incubation step is required if a secondary antibody is used to detect bound second affinity ligand in the assay system.

In another embodiment, using similar methods, the immobilized affinity ligand comprises a first affinity ligand recognizing an epitope common to both tsIL-2Rα and sIL-2Rα (and/or IL-2Rα; e.g., murine anti-human IL-2Rα antibody), and the second affinity ligand comprises an affinity ligand which binds specifically to tsIL-2Rα (e.g., not to sIL-2Rα nor IL-2Rα; e.g., anti-tsIL-2Rα monoclonal antibody). The second affinity ligand may be conjugated to a detectable moiety, or a secondary antibody used to detect the second affinity ligand may be conjugated to a detectable moiety. The immunoassay may further include a range of known concentrations of recombinantly produced tsIL-2Rα, or immunopurified tsIL-2Rα from cells grown in culture, or synthesized peptide having one or more tsIL-2Rα-specific

epitopes (See Example 4), to provide a standard curve for quantifying the level of tsIL-2Rα detected in the sample of body fluid. In providing a microtiter plate with wells containing immobilized affinity ligand, sites in the well which are not bound by immobilized affinity ligand may be blocked by a blocking agent, known to those skilled in the art, to prevent non-specific adsorption of the antigen and/or second antibody to the reaction surface.

6.2 Applications of the measurement of the tsIL-2Rα

A. Detecting metastatic cells, and assay kits

Because of the difficulties that metastases present in terms of diagnosis and treatment of an individual having metas-tases, a method for detecting metastatic cells is desirable. As described above, because the level of tsIL-2Rα can correlate with the presence and/or amount of metastatic cells, levels of tsIL-2Rα in a body fluid of an individual may provide an accurate prognosis for the development of metastases in that individual. Likewise, because tsIL-2Rα levels correlate with the presence of metastases, particularly in the absence of detectable primary tumor, levels of tsIL-2Rα in a body fluid of an individual may provide an accurate prognosis for the development of metastases in that individual (i.e., increasing levels of ts-IL2Rα in an individual with metastases is associated with a poorer prognosis); and/or may be an indicator that the individual has residual primary tumor that produces tsIL-2Rα in an early stage of the meta-static process. A method for detecting the metastatic cells comprises measuring the tsIL-2Rα levels in the appropriate body fluid(s) (i.e., depending on the tumor tissue type) using a clinical diagnostic kit for determining tsIL-2Rα

expression. An appropriate range of values for tsIL-2Rα may be established by a clinician without undue experimentation. The levels may vary depending on the tumor tissue type, the sample of body fluid analyzed, the stage of progression of metastatic disease, the stage of development of the metastasis process, and the metabolism and health of the individual. Such a clinical diagnostic kit can comprise, for example, reagents and reaction vessels for an immunoassay.

An assay kit for detecting the presence or absence of tsIL-2Rα in a sample of body fluid contains one or more affinity ligands that facilitates determination of tsIL-2Rα that may be present in the sample analyzed. In one embodiment, the assay kit comprises an affinity ligand that binds specifically to tsIL-2Rα (e.g., not to sIL-2Rα nor IL-2Rα; e.g., anti-tsIL-2Rα monoclonal antibody). In another embodiment, the assay kit may comprise two affinity ligands: a first affinity ligand which binds specifically to tsIL-2Rα (and not to sIL-2Rα nor IL-2Rα; e.g., anti-tsIL-2Rα monoclonal antibody); and a second affinity ligand which binds to an epitope shared by tsIL-2Rα with sIL-2Rα and/or IL-2Rα (e.g., an anti-human IL-2Rα antibody recognizing and binding to an epitope within the first approximately 240 amino acids of SEQ ID NO:1). In the case of two affinity ligands, one affinity ligand may be used to capture the appropriate molecule(s) for which it has binding affinity, whereas the other affinity ligand may be used to detect the molecule(s) for which it has binding affinity. An assay kit according to the present invention may further comprise one or more controls to be used in the assay system. For example, a positive control may comprise a solution of a detectable amount of tsIL-2Rα. A negative control may comprise a solution in which tsIL-2Rα is absent, or is present in an

amount below the level of detection in the assay system. The assay kit according to the present invention may further comprise one or more standards; wherein each standard contains a known amount of tsIL-2Rα. The one or more standards can be used to correlate the amount of detectable moiety detected from the assay process to an amount of tsIL-2Rα detected in a tested sample. The assay kit may further comprise one or more reagents used in the immunoassay process (e.g., a physiologically acceptable solution/ buffer); and/or instructions for use of the assay kit and components; and optionally, other accessories useful in carrying out the methods of the present invention.

A method for detecting the presence or absence of tsIL-2Rα, in a body fluid of an individual, comprises: (a) obtaining a sample of body fluid from the individual; (b) contacting the sample with an affinity ligand which binds to an epitope on tsILR2Rα; and (c) detecting the amount of tsIL-2Rα bound to the affinity ligand; wherein the lack of detectable amounts tsIL2-Rα is indicative of the absence of tsIL-2Rα in detectable amounts in the body fluid; and wherein the detection of IL-2Rα is indicative of the presence of tsIL-2Rα in the body fluid. As indicated previously herein, the method may be performed in a number of ways, depending on if the affinity ligand is directly conjugated to a detectable moiety, or if a secondary antibody is used as a conjugate, and whether a substrate is needed for detection of the conjugate.

B. Determining the stage of malignant disease

In considerations for treatment choices for a patient having a non-lymphoid tumor or metastases thereof, a method for determining the stage or advancement of that patient's cancer is desirable. As described above, because

tsIL-2Rα levels correlate with the tumor volume of a primary tumor which is producing metastatic cells, or of the number of metastatic cells, levels of tsIL-2Rα in a body fluid of a patient may provide an accurate indicator of the progression of malignant (including metastatic) disease in that patient. A method for determining the stage of progression (including development) of metastatic disease (e.g., a method of using tsIL-2Rα as a prognostic marker) may involve periodic measurements of tsIL-2Rα levels in the appropriate body fluid(s) (i.e., depending on the tumor tissue type) using an assay kit for determining tsIL-2Rα amounts. It will be apparent that the same type of body fluid need be used in successive measurements, or that if different body fluid types are used, they are known to contain similar amounts of tsIL-2Rα. The measured levels (amounts) of tsIL-2Rα are then compared to determine if there is an increase in successive measured levels of tsIL-2Rα (an indicator of advancing stages, and a poorer prognosis), a decrease in successive measured levels of tsIL-2Rα (an indicator of a decrease in malignant disease), or a constant level of approximately the same amounts in successive measured levels of tsIL-2Rα (maintaining the same stage of malignant disease).

Accordingly, a method of using tsIL-2Rα as a prognostic marker in an individual known to have metastatic cells comprises:

(a) obtaining a first sample of body fluid from the individual;

(b) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(c) obtaining a second sample of body fluid from the individual;

(d) testing the first sample by determining the amount

of tsIL-2Rα present in the sample;

(e) comparing the amount of tsIL-2Rα detected in the first sample to the amount of tsIL-2Rα detected in the second sample. A change in the amount of tsIL-2Rα detected in the first sample as compared to the second sample or subsequent samples of body fluid may be used as a marker for prognosis. For example, a decrease in the amount of tsIL-2Rα detected in the second sample or subsequent samples, as compared to amount in the first sample, may be an indicator prognosing advancement of the metastatic disease process or advancement in the development and/or progression of metastases.

C. Use of tsIL-2Rα as a tumor response marker

Because tsIL-2Rα levels correlate with tumor volume, tsIL-2Rα levels may be used as a tumor response marker to monitor a patient's response to anticancer therapy, whether the anticancer therapy comprises one or more of chemotherapy, radiation therapy, immunotherapy or surgical removal. Successive measurements of tsIL-2Rα can be made before treatment of the patient begins, and after one or more treatments of the patient; and the measurements are then compared. It will be apparent that the same type of body fluid need be used in successive measurements, or that if different body fluid types are used, they are known to contain similar amounts of tsIL-2Rα. Responsive of the tumor to anticancer therapy (i.e., a decrease in the tsIL-2Rα levels following therapy as compared to tsIL-2Rα levels prior to therapy) can be used for determining a dosage regimen and treatment schedule for an individual patient. An tumor response marker as such for determining a dosage regimen and treatment schedule would be particularly desirable in cases where a patient develops adverse side

effects as a result of anticancer therapy. A method for using tsIL-2Rα levels as a tumor response marker for monitoring efficacy of anticancer therapy involves periodic measurements of tsIL-2Rα levels in the appropriate body fluid(s) (i.e., depending on the tumor tissue type) using a clinical diagnostic kit for determining tsIL-2Rα expression. The measured levels of tsIL-2Rα levels are then compared to determine if there is an increase or the same level of tsIL-2Rα post-treatment as compared to the tsIL-2Rα level either before treatment and/or before a prior regimen of treatment (an indicator of lack of tumor responsiveness to anticancer therapy), or a decrease in the level of tsIL-2Rα post-treatment as compared to the tsIL-2Rα level either before treatment and/or before a prior regimen of treatment(an indicator of tumor responsiveness to anticancer therapy). One skilled in the art would appreciate that for this method, the time period between anticancer therapy and subsequent measurements of tsIL-2Rα would vary depending on the mode and frequency of therapy.

Accordingly, a method of using tsIL-2Rα as a tumor response marker for monitoring efficacy of anticancer treatment of an individual comprises:

(a) obtaining a first sample of body fluid from the individual;

(b) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(c) obtaining a second sample of body fluid from the individual;

(d) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(e) comparing the amount of tsIL-2Rα detected in the first sample to the amount of tsIL-2Rα detected in the second

sample; wherein the first sample was obtained from the individual at a time selected from the group consisting of prior to anticancer treatment of the individual, and after a regimen of anticancer treatment of the individual that precedes a subsequent regimen of anticancer treatment after which the second sample is obtained (e.g., the second sample is obtained after a successive regimen of anticancer treatment). A change in the amount of tsIL-2Rα detected in the first sample as compared to the second sample or subsequent samples of body fluid may be used as a marker for response to metastases or the metastatic process to treatment. For example, a decrease in the amount of tsIL-2Rα detected in the second sample or subsequent samples, as compared to amount in the first sample, may be an indicator that the anticancer treatment may have some efficacy against the metastases or the metastatic process; whereas an increased amount may indicate the lack of responsiveness of the metastases or the metastatic process to the anticancer treatment.

EXAMPLE 7

It will be apparent that nucleotide sequences encoding tsIL-2Rα may be operatively linked to a promoter in a vector for expressing the tsIL-2Rα when the resultant recombinant vector is introduced into a host cell. Thus, a recombinant vector for expressing tsIL-2Rα may be used to produce tsIL-2Rα for purposes of diagnostic (e.g., production of tsIL-2Rα antibodies) and/or therapeutic uses. As known to those skilled in the art, a vector is a nucleic acid molecule used as a vehicle for introducing into and expressing in a host cell a gene or nucleic acid sequence of interest. As known to those skilled in the art, such vectors may include, but are not limited to, plasmids, phage

vectors, viruses, and retroviruses. The features of a vector which make it useful in the present invention include that it have a selection marker for identifying vector which has inserted therein a nucleic acid sequence encoding tsIL-2Rα; restriction sites to facilitate cloning of a nucleic acid sequence encoding tsIL-2Rα; and the ability to enter and/or replicate in host cell. Depending on the desired form of recombinant tsIL-2Rα, host cells for expression may include well known eukaryotic cells (e.g., mammalian cells, insect cells, animal cell lines, human cells, human cell lines, plant cells) or prokaryotic cells (e.g., yeast, fungi, bacteria). The vector may further comprises one or more control elements which is operatively linked to a nucleic acid sequence encoding tsIL-2Rα in a manner permitting expression (e.g., a promoter) of tsIL-2Rα, or expression (promoter) and upregulation (enhancer) of expression of tsIL-2Rα. The choice of the control element, which is operatively linked to the nucleic acid sequence encoding tsIL-2Rα in the vector, depends on factors which may include, but are not limited to, the host cell system used for expression, the desired level of expression, whether expression is to be inducable or repressable, and the type of vector used. For example, promoters which can be used in prokaryotic systems are known in the art to include the lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, P_R and P_L promoters, T3, T7, and the like. Promoters which can be used in eukaryotic systems include, but are not limited to, CMV promoter, SV40 promoter, RSV promoter, HSV thymidine kinase promoter, metallothionein-I promoter, and the like. The selection of the appropriate vector, control element(s), and host cells for the expression of a molecule like tsIL-2Rα is well within the level of ordinary skill in the art.

A recombinant vector, containing a nucleic acid sequence encoding tsIL-2Rα for expression, is introduced into the desired host cells for expression. Depending on the host cells chosen, the recombinant vector may be introduced using a method that may include transformation, transfection, infection, or electroporation. The host cells containing the recombinant vector may then be grown in suitable medium and under suitable conditions for growth, and then selected and screened for. Selection and screening may be accomplished by methods known in the art such as detecting the expression of a marker gene (e.g., drug resistance marker) present in or encoded by the vector, immunoscreening for production of tsIL-2Rα epitopes, or probing for vector nucleic acid sequences encoding tsIL-2Rα by using one or more oligonucleotides for hybridizing to and identifying tsIL-2Rα encoding sequences. Host cells containing the recombinant vector may then be grown amounts and in suitable medium and under suitable conditions for growth, to achieve a sufficient amount of tsIL-2Rα which can then be harvested from the host cell culture system. The selection of the appropriate growth medium, culture conditions, and length of process for a sufficient amount of tsIL-2Rα to be produced for harvesting will depend on such factors as the host cell system used, and the vector used; and is well within the level of ordinary skill in the art. In host cells systems in which the tsIL-2Rα produced is shed or secreted from the host cells, the tsIL-2Rα may be recovered from the culture medium by any one of several methods known in the art to recover recombinant molecules including affinity chromatography, size exclusion, ion exchange chromatography, and magnetic bead separation.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, various modifications will become apparent to those skilled in the related arts from the foregoing description and figures. For example, tsIL-2Rα may be found in human malignancies other than solid, non-lymphoid tumors, and thus may serve as indicia for diagnostics, prognostics, and staging of such other malignancies. Such modifications are intended to be included within the spirit of this application.

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleotide sequence, wherein the nucleotide sequence encodes a soluble IL-2Rα produced by metastatic cells, and wherein the nucleotide sequence encodes an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

2. A recombinant vector containing the nucleic acid molecule according to claim 1, wherein the nucleic acid molecule is operatively linked to one or more control elements for expression.

3. A host cell containing the vector according to claim 2.

4. A method for producing a protein, wherein the protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7; said method comprising:
(a) growing a host cell according to claim 3 in growth medium for a sufficient time, and under suitable conditions, to produce a recoverable quantity of the protein secreted into the growth medium; and
(b) recovering the protein from the growth medium.

5. A composition for the nucleic acid amplification of a nucleic acid molecule encoding a soluble IL-2Rα produced by metastatic cells, wherein the composition comprises at least one oligonucleotide, and wherein the at least one oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

6. An isolated and purified protein comprising a soluble IL-2Rα produce by metastatic cells, wherein the protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

7. A method for detecting the presence or absence of tsIL-2Rα in a body fluid of an individual, said method comprises:

(a) obtaining a sample of body fluid from the individual;

(b) contacting the sample with an affinity ligand which binds to an epitope on tsILR2Rα; and

(c) detecting the amount of tsIL-2Rα bound to the affinity ligand;

wherein the lack of detectable amounts tsIL2-Rα is indicative of the absence of tsIL-2Rα in detectable amounts in the body fluid, and wherein the detection of IL-2Rα is indicative of the presence of tsIL-2Rα in the body fluid.

8. A method of using tsIL-2Rα as a prognostic marker in an individual known to have metastatic cells, said method comprises:

(a) obtaining a first sample of body fluid from the individual;

(b) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(c) obtaining a second sample of body fluid from the individual;

(d) testing the first sample by determining the amount of tsIL-2Rα present in the sample; and

(e) comparing the amount of tsIL-2Rα detected in the first sample to the amount of tsIL-2Rα detected in the second sample.

9. A method of using tsIL-2Rα as a tumor response marker for monitoring efficacy of anticancer treatment of an individual, said method comprises:

(a) obtaining a first sample of body fluid from the individual;

(b) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(c) obtaining a second sample of body fluid from the individual;

(d) testing the first sample by determining the amount of tsIL-2Rα present in the sample;

(e) comparing the amount of tsIL-2Rα detected in the first sample to the amount of tsIL-2Rα detected in the second sample;

wherein the first sample was obtained from the individual at a time selected from the group consisting of prior to anticancer treatment of the individual, and after a regimen of anticancer treatment of the individual that precedes a subsequent regimen of anticancer treatment after which the second sample is obtained.

10. An assay kit for detecting the presence or absence of tsIL-2Rα in a sample of body fluid, wherein the assay kit comprises an affinity ligand that binds specifically to tsIL-2Rα, and not to sIL-2Rα nor IL-2Rα.

11. The assay kit according to claim 10, wherein the affinity ligand comprises an anti-tsIL-2Rα monoclonal antibody.

12. The assay kit according to claim 10, further comprising a second affinity ligand that recognizes and binds to an epitope shared by tsIL-2Rα and a molecule selected from the group consisting of sIL-2Rα, IL-2Rα, and a combination thereof.

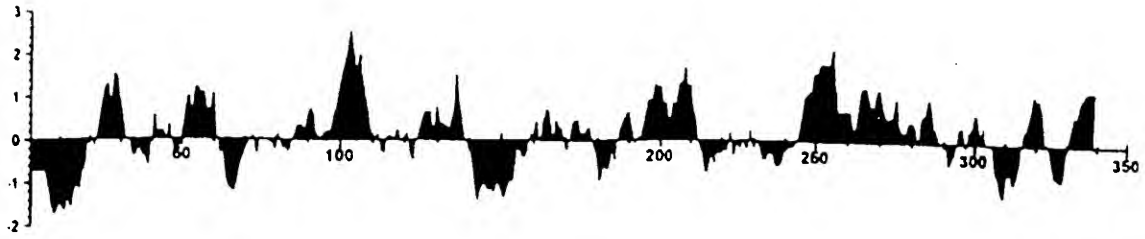
13. The assay kit according to claim 12 wherein the second affinity ligand comprises an anti-human IL-2Rα antibody that recognizes an epitope within the first approximately 240 amino acids of SEQ ID NO:1.

14. The assay kit according to claim 10, further comprising one or more controls to be used in the assay process.

15. The assay kit according to claim 14, wherein the one or more controls is selected from the group consisting of a positive control, a negative control, and a combination thereof; wherein the positive control comprises a solution containing a detectable amount of tsIL-2Rα; and wherein the negative control comprises a solution in which tsIL-2Rα is absent, or is present in an amount below the level of detection.

16. The assay kit according to claim 10, further comprising one or more standards; wherein each standard contains a known amount of tsIL-2Rα.

1/2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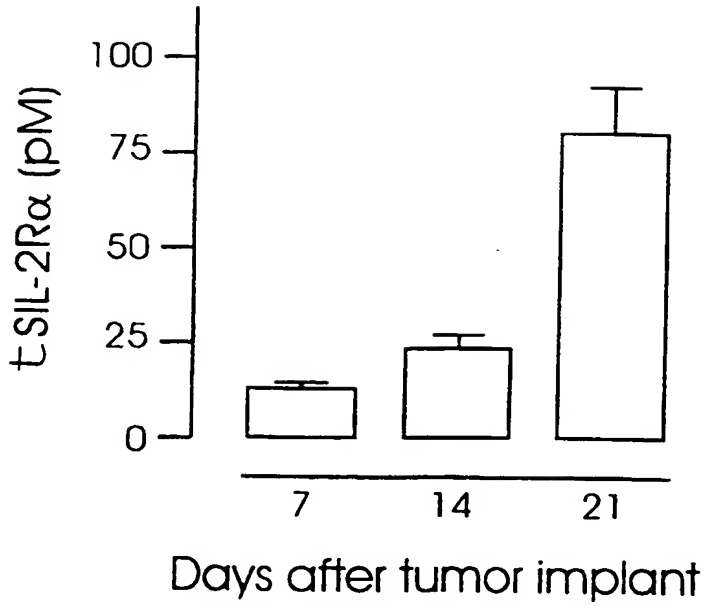
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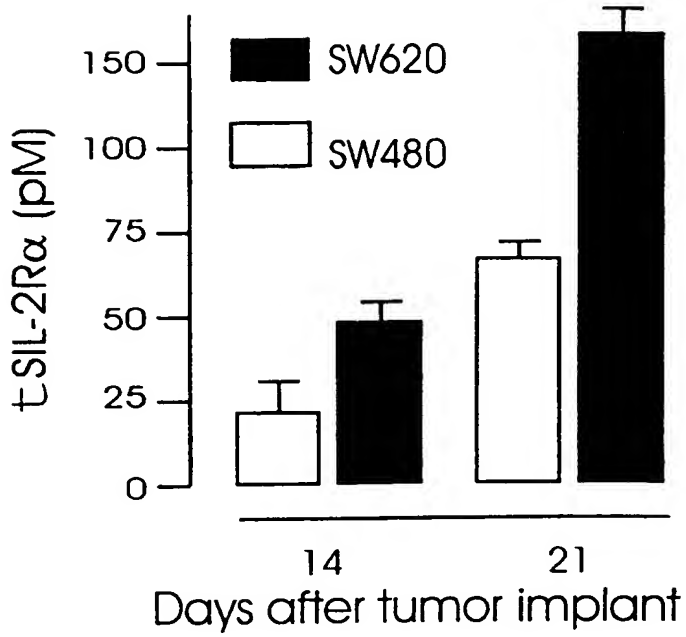
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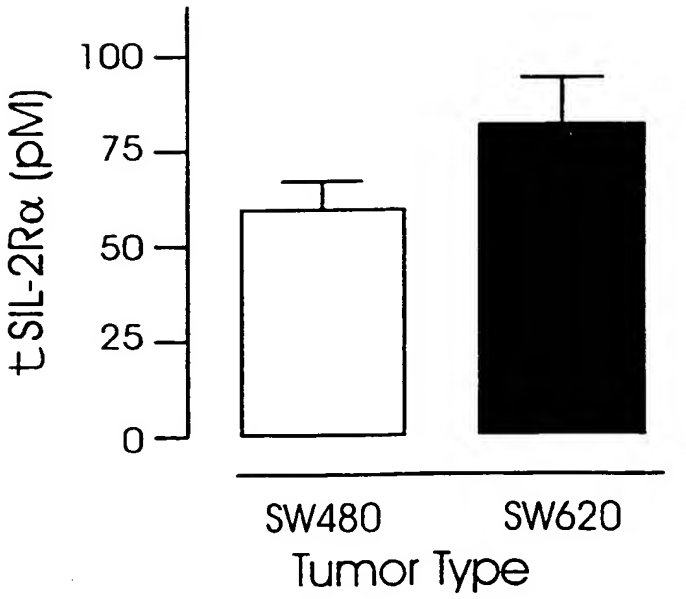
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FIG. 4

SEQUENCE LISTING

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<120> Methods and compositions using tumor specific soluble interleukin-2 receptor alpha molecules

<130> CLIO-28

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04222

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 23.4; 435/69.1, 69.5, 6, 7.1, 7.2, 252.3, 320.1; 530/350, 351; 514/2, 8, 12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,707,443 A (NELSON et al) 17 November 1987, see claims.	7-16
X	US 4,816,565 A (HONJO et al) 28 March 1989, see examples.	7-16
X	US 5,352,772 A (SMITH, K) 04 October 1994, see examples	7-16
X	US 5,449,756 A (TANIGUCHI et al) 12 September 1995, see examples.	7-16
X	US 5,356,795 A (LEONARD et al) 18 October 1994, see examples.	7-16
X	US 5,510,259 A (SUGAMURA et al) 23 April 1996, see examples.	7-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 JULY 1999	Date of mailing of the international search report 22 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GARNETTE D. DRAPER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Internationai appiication No.
PCT/US99/04222

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,856,140 A (SHIMAMURA et al) 05 January 1999, see examples.	7-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04222

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-6
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04222

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/00, 15/11, 1/00, 15/03, 15/63; C12Q 1/68; G01N 33/50; C07K 1/00, 14/715; A61K 38/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.5, 23.4; 435/69.1, 69.5, 6, 7.1, 7.2, 252.3, 320.1; 530/350, 351; 514/2, 8, 12

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE
2. Where no meaningful search could be carried out, specifically:

Claims 1-6 are directed to protein and nucleic acid products that are defined in terms of their amino acid or nucleic acid sequences. However, applicants have failed to provide a disk and paper copy of these sequences that are free of errors. The disk that was submitted and process by the STIC on 13 May 1999 contained errors.